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Treatment of Dogs with Compensated Myxomatous Mitral Valve Disease with Spironolactone – a Pilot Study

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Short title: Spironolactone in compensated MMVD

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Treatment of Dogs with Compensated Myxomatous Mitral Valve Disease with Spironolactone – a Pilot Study

Abstract

Objectives: Spironolactone improves outcome in dogs with advanced myxomatous mitral valve disease (MMVD). Its efficacy in preclinical MMVD is unknown. Hypothesis; administration of spironolactone to dogs with compensated MMVD demonstrating risk factors for poorer prognosis will decrease the rate of disease progression. Aim; to provide pilot data to evaluate preliminary effects and sample size calculation for a definitive clinical trial.

Animals: Twenty-five client-owned dogs with MMVD with at least one of the following; left atrial-to-aortic ratio (LA:Ao) ≥1.5, normalized left ventricular internal diameter in diastole (LVIDdN) ≥1.6), N-terminal pro-B-type natriuretic peptide (NT-proBNP) >550pmol/L, cardiac troponin I (cTnI) >0.025ng/mL.

Methods: Prospective, single-center, equally randomized, placebo-controlled, double-blinded, parallel grouped pilot study. No dogs were receiving medications for cardiac disease prior to enrolment.

Results: Twelve dogs received placebo; 13 received spironolactone. One dog in the spironolactone group died suddenly, 1 developed congestive heart failure and 2 received suboptimal spironolactone doses. At enrolment NT-proBNP was significantly higher in the spironolactone group (P=0.005). LA:Ao (P=0.002) and LVIDdN (P=0.005) increased over time in the placebo group, but not the spironolactone group; the change did not differ significantly between groups. The change in biomarker concentrations did not differ significantly between groups; there was a tendency towards an increase in NT-proBNP over

time in the placebo group. Enrolment of 76 dogs would be necessary to demonstrate a difference in the change in LA:Ao over 6 months between groups.

Conclusions: preliminary results support undertaking a larger clinical trial of treatment of dogs with preclinical MMVD with spironolactone.

Keywords: preclinical disease, therapy, canine

Abbreviations:

ACVIM	American College of Veterinary Internal Medicine
Ao	Aorta
CHF	Congestive heart failure
CKCS	Cavalier King Charles spaniel
cTnI	Cardiac troponin I
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
LA	Left atrium
LA:Ao	Ratio of left atrial to aortic root diameter
LVIDd	Left ventricular internal dimension in diastole
LVIDd/ LVFWd	Ratio of left ventricular end-diastolic dimension to left ventricular free wall thickness in diastole

LVIDdN	Left ventricular end-diastolic dimension normalized for body weight
LVIDs	Left ventricular internal dimension in systole
LVIDsN	Left ventricular end-systolic dimension normalized for body weight
LVFWd	Left ventricular free wall thickness in diastole
MMVD	Myxomatous mitral valve disease
NSAID	Non-steroidal anti-inflammatory drug
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PCV	Packed cell volume
UAC	Urinary aldosterone to creatinine ratio

63

Introduction

Myxomatous mitral valve disease (MMVD) is the commonest cause of cardiovascular disease in the dog [1, 2]. Valvular degeneration results in regurgitation of blood, leading to progressive volume overload of the left atrium and ventricle, which compensate by eccentric hypertrophy [3]. The American College of Veterinary Internal Medicine (ACVIM) classification system for MMVD describes four disease stages, from A (at risk) to D (decompensated congestive heart failure) [4]. The compensated, preclinical stage of the disease (stage B) is subdivided according to the absence (stage B1) or presence (stage B2) of evidence of compensatory hypertrophy, identified on the basis of chamber enlargement on radiography or echocardiography. The rate of progression of the disease is variable and the development of heart failure is not an inevitable consequence of MMVD [5]. However, even with optimal medical therapy, once dogs with MMVD develop congestive heart failure (CHF), median survival is approximately 270 days [6]. Pimobendan has been shown to delay the onset of congestive heart failure in dogs with ACVIM class B2 MMVD [7]. Further therapeutic strategies, targeting different mechanisms of disease progression, might provide additional clinical benefit in dogs at risk of disease progression.

The identification of dogs at greatest risk of progression of MMVD is important in a disease with such variability of outcome. Evidence of cardiac remodelling, such as increased echocardiographic measurements of left atrial [5] and left ventricular size, [8] are associated with decreased survival times in dogs with MMVD. Increases in secondary markers of myocardial stress and injury, specifically, serum N-terminal pro-B-type natriuretic peptide (NT-proBNP) > 524 pmol/L and/ or serum cardiac troponin I (cTnI) > 0.025 ng/mL, are also associated with poorer outcomes [9]. Increased left atrial size is associated with an increased risk of disease progression for dogs in ACVIM class B [10].

Activation of the renin-angiotensin-aldosterone system is important in the pathophysiology

of cardiac remodelling in canine MMVD [11]. Via its actions on the mineralocorticoid receptor, aldosterone promotes fluid retention, leading to volume-overload and stimulates myocardial fibrosis [12]. Urinary aldosterone to creatinine ratio (UAC) is associated with the rate of change of left ventricular size in dogs with MMVD, suggesting that aldosterone production increases during periods of active remodelling [13]. Spironolactone is a mineralocorticoid receptor antagonist that has been shown to prolong survival times in dogs with advanced MMVD and CHF secondary to MMVD, when given in combination with standard therapy [14]. The use of spironolactone in dogs with compensated MMVD has not been reported, although a study investigating its effects in combination with benazepril is currently ongoing.

We hypothesized that chronic oral administration of spironolactone to dogs with compensated MMVD demonstrating risk factors known to be associated with decreased survival times (increased left atrial and ventricular size and increased serum NT-proBNP and cTnI), not receiving any other cardiovascular medications, would result in decreased rates of change of these risk factors over time. The aim of the study was to provide pilot data to evaluate preliminary effects, drug safety and to calculate the number of dogs needed for a definitive clinical trial.

Animals, Materials and Methods

Study Design

The design of this pilot study was single-centre, prospective, equally randomized, double-blinded, parallel-grouped and placebo-controlled. Each dog participated in the study for a period of six months. The study was conducted in the United Kingdom. The study was approved by the Royal Veterinary College Ethical Committee and specific informed owner

113 consent was obtained (unique reference number 2010 1039).

114

115 *Dogs*

116 Client-owned dogs of a variety of breeds with echocardiographically-confirmed MMVD were
117 prospectively recruited between December 2010 and December 2013 from those already
118 enrolled in a longitudinal study of canine MMVD in first opinion practice [8]. Dogs were
119 referred to the longitudinal study by the veterinarians at 2 London-based first opinion
120 practices after detection of a murmur consistent with mitral regurgitation at any stage in the
121 natural history of the disease. Echocardiography was performed to confirm the diagnosis of
122 MMVD and to exclude the presence of other cardiac diseases. Diagnosis of MMVD was on
123 the basis of characteristic abnormalities of the valve leaflets (thickening, prolapse, or both)
124 and evidence of regurgitant flow across the valve detected by Doppler. Dogs with any other
125 cardiac disease or clinically relevant organ-related or systemic disease were not enrolled in
126 the longitudinal study.

127 To be eligible for inclusion in the present study, a dog had to have echocardiographic
128 evidence of MMVD, defined as above, and demonstrate at least one risk factor for disease
129 progression (evidence of cardiomegaly (defined as left atrial to aortic ratio (LA:Ao) ≥ 1.5
130 and/ or left ventricular end-diastolic dimension, normalized for body weight (LVIDdN)) >
131 1.6 [8], serum NT-proBNP > 550pmol/L [9] and/ or serum cTnI > 0.025 ng/mL [9]. During
132 the screening process, but not during the experimental phase, serum NT-proBNP was
133 measured using the first-generation version of a commercially-available enzyme-linked
134 immunosorbent assay (ELISA).[c]

135 Dogs were excluded from the study if they had any of the following: evidence of any
136 congenital or acquired cardiac disease other than MMVD; evidence of kidney disease,

hypoadrenocorticism (on the basis of historical, physical examination and routine biochemical findings; ACTH stimulation tests were not performed), hyperkalemia, or hypernatremia; current or previous clinical signs of congestive heart failure or current medical therapy for cardiac disease. The summary of product characteristics for Prilactone recommends that dogs treated concomitantly with spironolactone and non-steroidal anti-inflammatory drugs (NSAIDs) be correctly hydrated,[d] and so it was recommended that dogs should not receive NSAIDs, although this was not an absolute exclusion criterion.

Randomization

Randomization was by patient (dog). The study was initially designed to recruit 20 dogs. Prior to the enrolment phase, a numbered list of 20 random group assignments (either group A or group B) was compiled by drawing assignments from a hat. Dogs were assigned to groups according to the order in which they were enrolled in the study. Prospectively determined, prognostic factor balance was achieved by minimization to ensure that the number of cavalier King Charles spaniels (CKCS) was equal in each group [15]; briefly, if, in the latter stages of the recruitment phase (from dog number 11 onwards), enrolling a CKCS to the next group assignment according to the randomization list would have resulted in unbalancing of the groups, then the dog was assigned to the alternative treatment group. No other variable was considered prior to enrolment. Data obtained from the first 20 dogs were analysed and reported in abstract form[e] and the decision made to recruit an additional 20 dogs to increase the statistical power of the study. An additional numbered list of 20 random assignments (either group C or group D) was compiled in the same way to allow recruitment of additional dogs to the study in a blinded fashion after treatment allocation of groups A and B was revealed at the time of previous data analysis.

Blinding

The investigators and owners were blinded to the treatment allocation. Assignment of enrolled dogs to treatment groups was performed by a veterinary nurse to conceal allocation from the investigators responsible for measurement of the variables of interest. Data for the first 20 dogs enrolled (groups A and B) was analyzed prior to recruitment of the additional dogs (groups C and D).[e] The blinding codes for the treatment groups were held by the sponsor until the time of each data analysis.

Trial medication

Spironolactone verum (Prilactone 10 mg tablets)[f] was administered orally at a target dose of 2 mg/kg SID, as per registered label instructions, and the dose adjusted to a suitable number of tablets. Placebo was administered PO according to the calculated daily dose for spironolactone verum tablets and adjusted to a suitable number of placebo tablets. The tablets and packaging of the verum and placebo were visually indistinguishable. Dogs received tablets (verum or placebo) orally once daily for 6 months, unless otherwise stated. The dose of the study medication was not adjusted during the study period. Additional appropriate medication was prescribed if clinical signs of cardiac failure developed during the study period. Participation was terminated if clinical signs of another significant medical condition occurred which required additional treatment or warranted euthanasia, or if adverse effects were observed which necessitated cessation of the therapy. A record was kept of other medication used. Compliance was monitored by counting the number of unused pills returned by the owner at the end of the study period. This number was compared with the expected number of pills remaining.

185

186 *Schedule of Events*

187 Prior to enrolment, serum biochemistry and electrolyte measurements were performed.

188 At baseline, enrolled cases underwent a full evaluation, comprising recording of the history,
189 measurement of systolic arterial blood pressure by Doppler sphygmomanometry,[g] physical
190 examination, blood sampling, electrocardiography (ECG) and echocardiography, in that
191 order. Electrocardiography was performed in right lateral recumbency and heart rate was
192 measured from a 60-second recording of lead II. Treatment was initiated with either verum or
193 placebo. Re-examinations were scheduled at day 14 and approximately 6 months after
194 inclusion. The tests performed at each study visit are summarized in Figure 1.

195

196 *Clinical Evaluation*

197 At inclusion, demographic characteristics (age, breed, sex and neutering status) were
198 recorded. Body weight and body condition score were recorded at each study visit.

199

200 *Blood sampling and Laboratory Analysis*

201 Blood was collected by jugular venepuncture into serum gel tubes and K3-EDTA-treated
202 tubes. Free-catch urine samples were collected. Samples were chilled at 4°C for up to 6 hours
203 before separation by centrifugation. Packed cell volume (PCV) was measured prior to
204 separation. Serum and urine samples were transported to a commercial laboratory for
205 measurement of routine biochemical parameters and electrolytes.[h] The remaining serum and
206 urine were stored at -80°C for batched analysis. Urinary aldosterone concentrations were
207 measured using a previously-validated, commercially-available radioimmunoassay [16]

following mild acid hydrolysis and extraction into ethyl acetate, as previously described [13]. Serum aliquots were transported to the same commercial laboratory on dry ice. Before analysis, the frozen serum was allowed to thaw slowly at room temperature. Concentrations of cTnI were measured using an ELISA[i] according to the manufacturer's instructions. The use of this assay has been previously validated for canine samples [17]. Concentrations of NT-proBNP were measured using the second-generation version of a previously-validated canine NT-proBNP ELISA[c] according to the manufacturer's instructions [18]. Serum biochemistry and electrolytes, NT-proBNP, cTnI, PCV and UAC were measured at the baseline visit. On day 14, PCV, serum biochemistry and electrolytes and UAC were measured. Full clinical evaluation, plus measurement of PCV, serum biochemistry and electrolytes, NT-proBNP, cTnI and UAC, was repeated at the 6 month time point, unless otherwise stated.

Echocardiography

Echocardiography was performed at baseline and at the 6 month visit. Echocardiographic examinations were performed by a single board-certified cardiologist (AB). Dogs were placed in right and then left lateral recumbency on an ultrasound examination table. The echocardiographic examination was performed using an ultra-sound unit[j] equipped with 2–4 MHz and 3–7 MHz phased array transducers and ECG monitoring. Standard imaging planes were digitally stored. Assessment of mitral valve structures was performed from the right parasternal long-axis view and the left apical 4-chamber view. The LA:Ao was measured from the right parasternal short axis view, as previously described [19]. Left ventricular internal diameters in systole and diastole (LVIDs and LVIDd, respectively) and wall thicknesses were measured from M-mode obtained from the right parasternal short axis view. LVIDs was normalized for body weight (LVIDsN) by the formula: LVIDs/ (body weight

[kg])$^{0.315}$[20]. LVIDd was normalized for body weight (LVIDdN) by the formula: LVIDd/ (body weight [kg])$^{0.294}$[20]. The ratio of LVIDd to left ventricular free wall thickness in diastole (LVFWd) was calculated (LVIDd/ LVFWd) as an indirect estimate of wall stress. Measurements were recorded from at least 3 cardiac cycles and the mean value used in subsequent analyses.

The primary outcome comparisons of the study were comparisons of the change in LA:Ao, LVIDdN and serum NT-proBNP and cTnI between groups over a 6 month period. Secondary outcome comparisons were comparisons of the change in other variables (PCV, serum urea, creatinine and electrolyte concentrations, UAC, LVIDsN, LVIDd/ LVFWd ratio, E wave velocity, E/A wave ratio, heart rate and body weight) between groups over the same 6 month period and between-group comparisons at different time points (baseline and 2 and 26 weeks after enrolment). Results are reported according to the Consolidated Standards of Reporting Trials 2010 guidelines for reporting parallel group randomized trials [21].

Statistical Analysis

Data were analyzed on an intention to treat basis and data from all dogs that were randomly assigned were included. Statistical analyses were performed using commercially-available software.[k] Data were assessed for normality graphically and by use of the Shapiro-Wilk test. Results are reported as mean ± standard deviations for normally distributed continuous variables or median [range] for non-normally distributed variables. Repeated measures linear mixed models with the random effect of subject (dog) were constructed to compare the change in variables over time, and a compound symmetry (co)variance structure was assumed between residuals of the same subject (dog) in the model. The effects of treatment group (spironolactone vs. placebo), time (treated as a continuous covariate) and interaction

between treatment group and time were included in the model. Residuals were assessed graphically for normality. Variables were logarithmically transformed if the residuals were not normally distributed. The assumption of homogeneity of variance was tested by plotting the predicted values against the residual values. Comparisons of continuous variables between groups at different time points were made using independent t-tests or Mann-Whitney U tests, as appropriate. Fisher's exact tests were used to compare proportions between groups at baseline. A value of $P \leq 0.05$ was considered significant. In view of the small sample size and the pilot nature of the study a value of $P < 0.1$ was considered to indicate a tendency towards significance. A sample size calculation was performed on the basis of the data obtained from the present pilot study using commercially-available software, assuming $\alpha = 0.05$ and $\beta = 0.2$ (i.e. power = 0.8).[1]

Results

Progress through the phases of the study is summarized in Figure 2. Twenty-five dogs with compensated MMVD diagnosed on the basis of echocardiographic findings were enrolled in the study. Due to the low rate of suitable dogs presenting to the larger longitudinal study, it proved impossible to recruit 40 dogs. Twenty-four dogs had LVIDdN > 1.6 and/ or LA:Ao > 1.5. Fifteen dogs had a previous measurement of serum cTnI > 0.025 pmol/L. Twenty-one dogs had a previous measurement of serum NT-proBNP > 550 pmol/L. All dogs met the inclusion criteria; 13 dogs demonstrated all three risk factors, 8 dogs demonstrated two of the risk factors and 4 dogs demonstrated one risk factor. Baseline characteristics of the two groups are presented in Table 1. There was no evidence for differences in age, body weight, gender or proportions of CKCS to other breeds between groups. Serum NT-proBNP concentrations were higher in the spironolactone treatment group compared with the placebo group ($P = 0.005$). At baseline there was a tendency for serum potassium ($P = 0.078$) to be

higher in the spironolactone treatment group compared with the placebo group. Group-wise comparisons were otherwise unremarkable. No evidence of kidney disease, hypoadrenocorticism, hyperkalemia, or hypernatremia was detected in any dog on serum biochemical and electrolyte analysis.

Twelve dogs (5 neutered females, 2 entire males and 5 neutered males) with ages ranging from 6.3 to 13.1 years and body weights ranging from 4.5 to 18.2 kg were assigned to receive placebo. These dogs comprised 7 CKCS, 2 mixed breeds and 1 each of bichon frisé, lurcher and toy poodle. No compliance problems, potential adverse drug reactions or adverse events were reported for dogs in this group. Two dogs had been receiving NSAIDs prior to, but not at the time of, recruitment. NSAID therapy was reinitiated by the primary veterinarian during the trial period in one of these dogs. NSAID therapy was initiated during the trial period in a third dog.

Thirteen dogs (2 neutered females and 11 neutered males) with ages ranging from 6.1 to 13.4 years and body weights ranging from 1.8 to 23.3 kg were assigned to receive spironolactone. These dogs comprised 8 CKCS, 2 cross-breeds and 1 each of bichon frisé, collie and Chihuahua. One dog in this group received a suboptimal dose of trial medication (1.3 mg/kg once daily) throughout the trial period, due to owner non- compliance. One dog was treated for seasonal allergic dermatitis during the trial period, during which time the trial medication was withdrawn for 10.6 weeks. Trial medication was reinstated following resolution of the dermatitis, which did not subsequently recur.

Two dogs in the spironolactone group suffered adverse events during the trial period (one dog developed congestive heart failure requiring medical management and one dog died suddenly). The dog that died suddenly was not known to be in congestive heart failure prior to death. There was no difference in the proportion of dogs experiencing adverse events between groups ($P = 0.480$). One dog had been receiving NSAIDs prior to, but not at the

time of, recruitment. NSAID therapy was reinitiated by the primary veterinarian during the trial period; this was the dog that died suddenly, although the death was considered to be unrelated to NSAID therapy.

Results of repeated measures linear model analyses for the primary outcome comparisons are summarized in Table 2. Residuals were not normally distributed for serum cTnI. Following logarithmic transformation of this variable the residuals were normally distributed. Because baseline serum NT-proBNP measurements were higher in the group receiving spironolactone, baseline measurements were included in the model as a covariate for this variable. No significant differences were detected for the change over time of any variable between groups. The change in serum NT-proBNP tended to be greater for the placebo group ($P = 0.087$). There was a tendency for serum NT-proBNP concentrations to increase in the placebo group, but not in dogs receiving spironolactone ($P = 0.073$) (Figure 3). Left atrial to aortic ratio ($P = 0.002$) and LVIDdN ($P = 0.005$) increased over time in the placebo group, but not in dogs receiving spironolactone (($P = 0.231$ and $P = 0.194$, respectively, Figures 4 and 5); however, in the absence of significant differences in the change over time between groups these findings should be interpreted with caution.

Results of repeated measures linear model analyses for the secondary outcome comparisons are summarized in Table 3. Residuals were not normally distributed for UAC, serum creatinine or E wave velocity. Following logarithmic transformation of these variables the residuals were normally distributed. There was a tendency for LVIDd/ LVFWd ratio to increase over time in the placebo group ($P = 0.070$), but not in dogs receiving spironolactone ($P = 0.315$). The change in LVIDd/ LVFWd ratio over time was not different between groups. There was a tendency for body weight to decrease in the dogs receiving spironolactone ($P = 0.066$), but not in the placebo group. The change in body weight was not different between groups.

Comparisons between groups at the 2 week time point are summarized in Table 4. No dogs were withdrawn from the study at this time point due to pharmacovigilance concerns. Urinary aldosterone to creatinine ratio was significantly higher in dogs receiving spironolactone than those receiving placebo (P = 0.006). There was a tendency for serum potassium to be higher in dogs receiving spironolactone than those receiving placebo (P = 0.098). There was no evidence of differences in any other variable between groups at this time point.

Comparisons between groups at the 6 month time point are summarized in Table 5. There was a tendency for serum NT-proBNP to be higher in dogs receiving spironolactone than those receiving placebo (P = 0.051). There was no evidence of differences in any other variable between groups at this time point.

A post-hoc sample size calculation was performed using the mean change in LA:Ao observed from these preliminary data, which suggested that a total sample of 76 dogs (38 per group) would be necessary to demonstrate a significant difference in the change in LA:Ao over 6 months between groups if one existed. However, use of the 95% confidence intervals for the change in LA:Ao for the sample size calculation suggested that the range of total sample size required to demonstrate a difference is 36 to 2936 dogs.

Discussion

The results of the present study suggest that a larger sample size (76 dogs) would be necessary to definitively test the hypothesis that spironolactone slows the rate of progression of preclinical MMVD. Although none of the between groups comparisons reached statistical significance, this pilot study is likely to be underpowered to demonstrate such differences. Four dogs receiving spironolactone failed to adhere to the protocol throughout the entire six month study period; one died suddenly, one developed congestive heart failure necessitating

the addition of other therapy, one received a suboptimal dose of medication and administration of trial medication was suspended in one dog for part of the trial period. This is likely to have further reduced the power of the study to detect differences between the groups. A post hoc sample size calculation was performed in order to inform the design of a subsequent, larger study that could be performed to determine whether a real treatment effect exists. Given the 95% confidence interval of the average change in LA:Ao, the number of dogs required could be as few as 36 or as many as 2936. Because of the intrinsic unreliability of such a sample size prediction on the basis of data from a relatively small population it is possible that there is no treatment effect, in which case no difference between groups would be demonstrated even if an infinite number of dogs was studied. No definitive conclusions regarding any effect of spironolactone on disease progression in dogs with MMVD can therefore be drawn on the basis of the results of this pilot study.

Echocardiographic measurements of cardiac size (LA:Ao and LVIDdN) increased over time in the placebo group but not in dogs receiving spironolactone, although no difference in the change over time was detected between groups. This suggests that performing a larger, definitive clinical trial is warranted to further investigate whether treatment with spironolactone slows disease progression in dogs with preclinical MMVD. Increasing LA:Ao and LVIDdN are known to be associated with reduced survival times in dogs with MMVD [22]. It is possible that decreasing the rate of increase of cardiac size might delay the onset of congestive heart failure, although large, long-term studies are required to investigate this hypothesis.

The radiographic indices of cardiac size in CKCS with compensated MMVD increase in a non-linear fashion with disease progression, reaching their maximal rate of change in the 9 months prior to the onset of congestive heart failure [23]. It was expected, therefore, that in this population of dogs with compensated MMVD and risk factors likely to be associated

with progression, echocardiographic indices of cardiac size would increase significantly over a six month period. The observation that LA:Ao and LVIDdN increased significantly in the placebo group suggests that this expectation was reasonable. Patients with MMVD that have higher NT-proBNP concentrations and greater heart size tend to have more advanced disease. Thus, since the group of dogs receiving spironolactone had significantly higher NT-proBNP concentrations at baseline it might have been expected that these dogs would have a more rapid rate of change than those in the placebo group. This might have contributed to the failure of the study to demonstrate a difference in the change over time in LA:Ao and LVIDdN. Future studies could use stratification of randomization of recruited cases by echocardiographic indices of heart size and/ or serum NT-proBNP concentrations to ensure that treatment groups are balanced with respect to these variables. In a small pilot study this approach was not feasible.

Urinary aldosterone to creatinine ratio was higher in dogs receiving spironolactone compared with placebo at the 2 week time point but not at the 6 month time point. Mineralocorticoid receptor blockade by spironolactone increases aldosterone secretion by stimulation of renin production [24]. It was expected that UAC would remain higher in the group receiving spironolactone throughout the study period. However, data were missing for this variable on 21/ 69 (30.4%) occasions on which it should have been measured due to failure to obtain urine samples. This would have decreased the power of the study to detect significant differences in UAC between treatment groups.

There was a tendency for serum potassium concentrations to be higher in dogs treated with spironolactone at baseline and the 2 week time point. Nevertheless, all measurements remained within the range of normal values and so this is not a source of pharmacovigilance concern nor has it been noted as a clinically significant adverse event in the published clinical trials involving spironolactone in dogs [25].

406 There was no evidence that the serum cTnI concentrations changed over time in either
407 treatment group. Rapid increases in serum cTnI concentrations only occur late in the course
408 of the disease, within the last 6 months of life of dogs that die due to MMVD [9]. A rapid
409 increase in serum cTnI would not, therefore, be expected in dogs in the compensated phase of
410 the disease.

411 Fractional shortening is usually increased in the compensated phase of MMVD, as the
412 presence of mitral insufficiency results in altered loading conditions. LVIDsN does not,
413 therefore, increase until the late stages of MMVD [22, 26]. In a similar fashion to serum
414 cTnI, it would not therefore be expected that LVIDsN would rapidly increase over a six
415 month period in this population of dogs.

416 Increased E wave velocities are indicative of increased ventricular filling pressures and the
417 volume of the regurgitant jet [26]. The results of the present study suggest that ventricular
418 filling pressures were not increasing and ventricular diastolic function was not declining in
419 either treatment group, probably reflecting the relatively early stage of the disease in this
420 population. E wave velocities >1.2 m/s have previously been shown to be associated with an
421 increased risk of disease progression in dogs with ACVIM class B MMVD; [10] the majority
422 of dogs in the present study had E wave velocities <1.2 m/s at the baseline visit.

423 This study has a number of limitations. Firstly, the sample size calculation suggests that on
424 the basis of our own data 76 dogs would need to be studied in order to demonstrate a
425 difference in the change in LA:Ao over 6 months between groups if one exists. This pilot
426 study was therefore underpowered to detect such a difference. We have therefore referred to
427 tendencies towards significance in the data with a more lenient P-value of < 0.1, while
428 recognizing the risks of a type I statistical error inherent in this approach. Secondly, no
429 consensus exists with regard to determining optimal cut-offs for echocardiographic evidence
430 of cardiomegaly in canine MMVD. In the present study, LA:Ao > 1.5 and / or LVIDdN >

1.6, were used to select dogs at risk of progression. These represent relatively liberal criteria, as the upper 95% confidence interval for LVIDdN is 1.85 [20]. However, in the present study these cut-offs were used only as inclusion criteria, in an attempt to identify dogs with more advanced compensated MMVD that were more likely to experience disease progression over a 6 month period. Previous studies have demonstrated that dogs above cut-off values lower than the value of 1.85 are at increased risk of cardiac related mortality [8]. Thirdly, increases in serum cTnI are not specific for cardiac disease,[27, 28] and so use of this marker as an inclusion criterion has limitations. Nevertheless, no dog was recruited solely on the basis of the cTnI measurement.

Fourthly, this study included a high proportion of CKCS. Urinary aldosterone to creatinine ratio has been shown to be higher in CKCS than non-CKCS breeds [13]. It is possible, therefore, that any benefit of mineralocorticoid receptor blockade might be greater in CKCS than other breeds. For this reason, the groups were balanced for numbers of CKCS by the minimization method; the trial was not, therefore, truly randomized. However, mineralocorticoid receptor antagonists have been shown to be beneficial in human patients, regardless of plasma aldosterone concentrations [29]. Large studies that allow for sub-analyses according to breed are necessary to investigate whether breed- specific effects of spironolactone exist.

Finally, allocation concealment would ideally have been performed externally rather than by a member of the regular clinic team. Inadequate allocation concealment may lead to selection bias and hence produce spurious results. In the present study, however, every attempt was made to maintain allocation concealment and therefore avoid bias of this nature.

Conclusions

455 The preliminary findings of this pilot study support undertaking a larger, definitive,
456 prospective, randomized, placebo-controlled, double-blinded clinical trial to further evaluate
457 the effect of spironolactone on disease progression in dogs with preclinical MMVD.

458

459 Footnotes

460 [c] Canine Cardiopet Nt-proBNP, IDEXX Laboratories, Westbrook, ME

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466 [f] CEVA Santé Animale, Libourne, France

467 [g] Park model 811 B, Perimed, Bury St. Edmonds, UK

468 [h] IDEXX Laboratories, Wetherby, UK

469 [i] Access Systems AccuTnI Assay, Beckman Coulter Inc., Fullerton, CA

470 [j] Acuson Cypress, Siemens Medical Solutions, Siemens House, Oldbury, Bracknell, UK

471 [k] IBM SPSS version 23, SPSS Inc., Chicago, IL

472 [l] GLIMMPSE version 2.2.4, University of Colorado, Denver, CO

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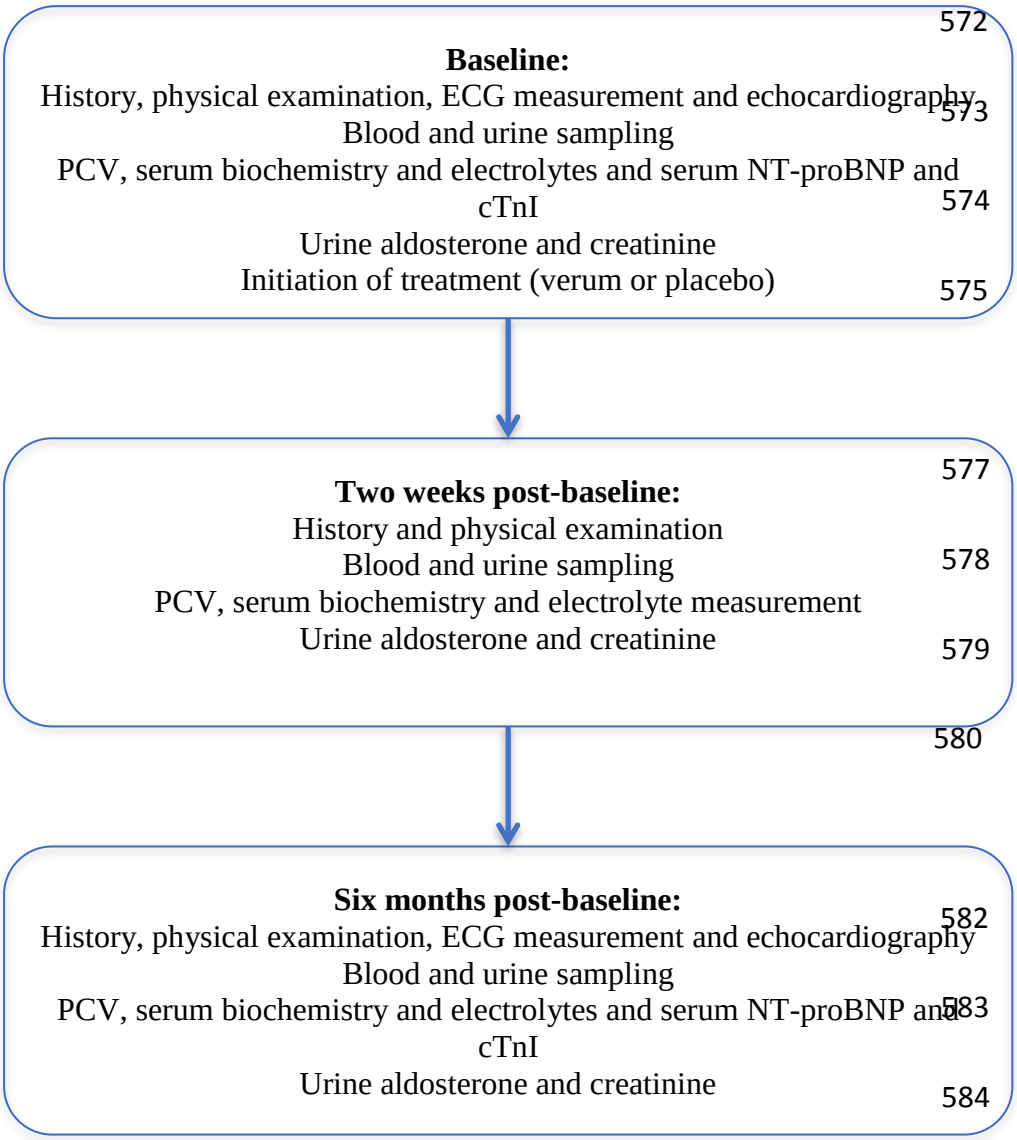
Figure 1: Summary flow diagram detailing the tests performed at each visit during the study period.



ECG, electrocardiogram; PCV, packed cell volume; NT-proBNP, N-terminal B-type natriuretic peptide; cTnI, cardiac troponin I.

589 Figure 2: Flow diagram of the progress through the phases of this parallel, randomized trial of
590 two groups. Analysis was on the basis of intention to treat.

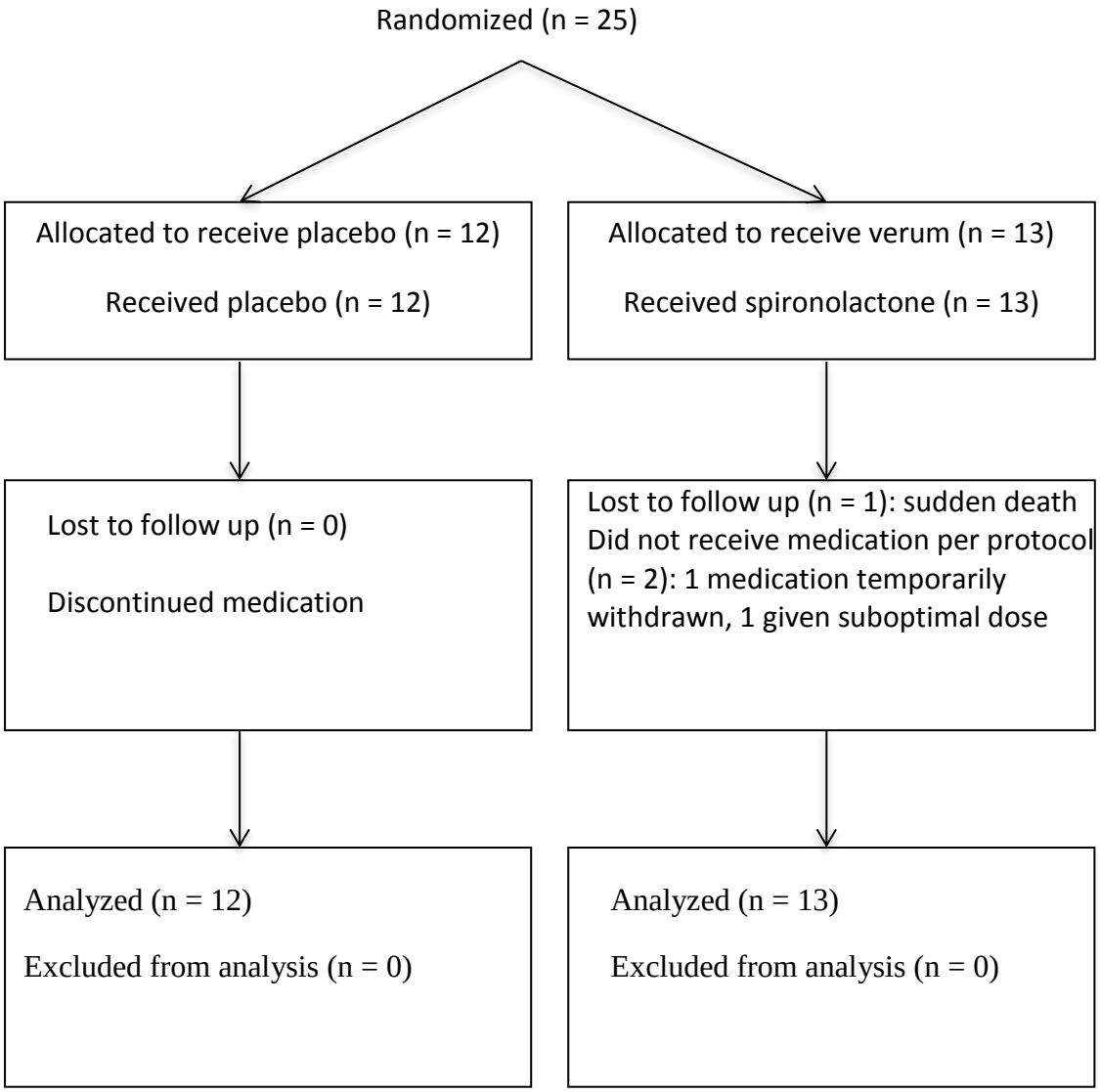
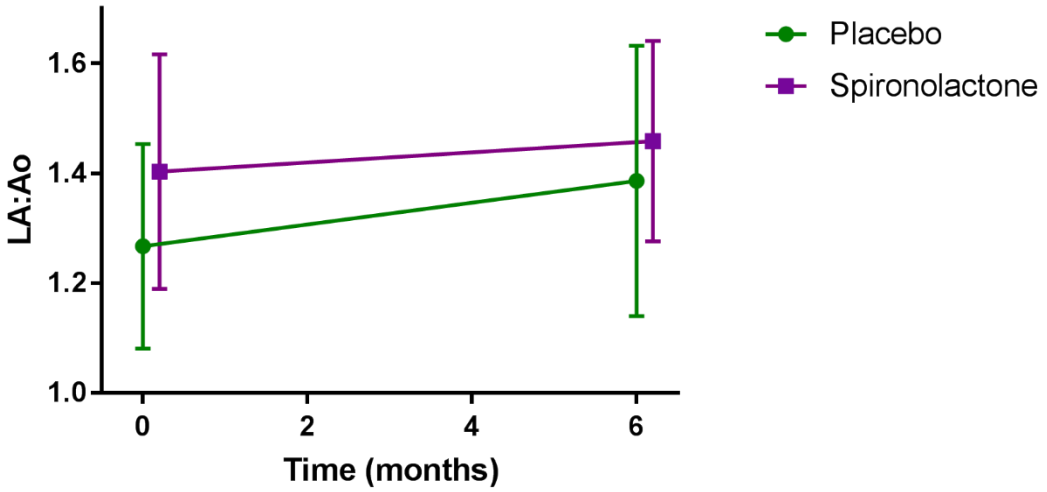


Figure 3: LA/Ao in dogs receiving placebo and those receiving spironolactone at baseline (n = 12 and 13, respectively) and at the 6 month time point (n = 12 and 12, respectively). The central tendency represents the mean and the error bars represent the standard deviation.



595 Figure 4: LVIDDN (cm/ [$kg^{0.294}$]) in dogs receiving placebo and those receiving
596 spironolactone at baseline (n = 12 and 13, respectively) and at the 6 month time point (n = 12
597 and 12, respectively). The central tendency represents the mean and the error bars represent
598 the standard deviation.

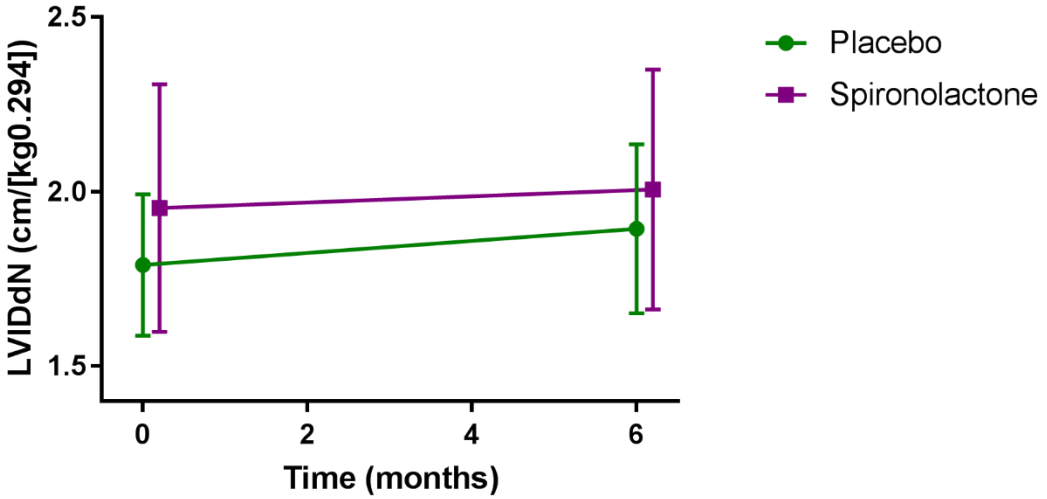


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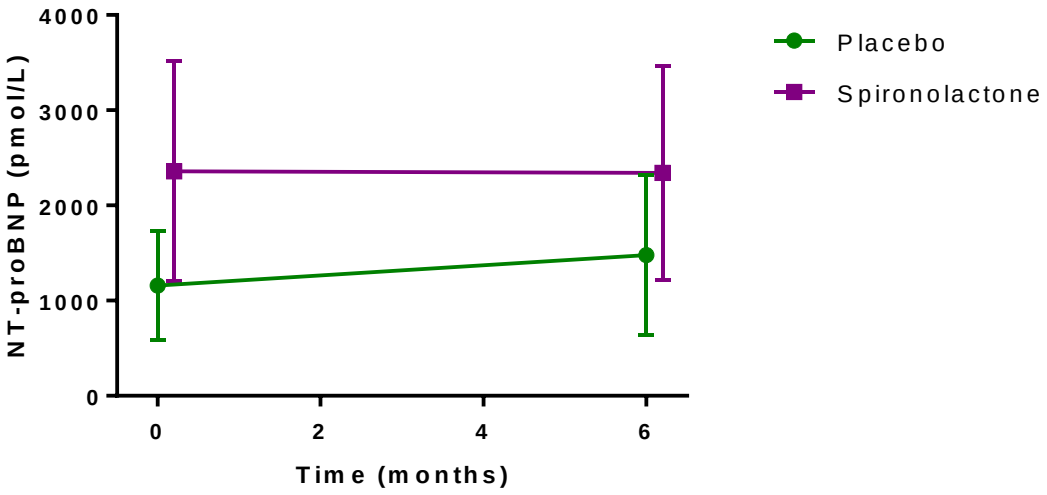
Figure 5: Serum NT-proBNP concentrations (pmol/L) in dogs receiving placebo and those receiving spironolactone at baseline (n = 12 and 13, respectively) and at the 6 month time point (n = 12 and 11, respectively). The central tendency represents the mean and the error bars represent the standard deviation.



607 Table 1: Comparisons of clinical, echocardiographic and biomarker data between dogs
608 receiving placebo and those receiving spironolactone at baseline.

Variable	Group receiving placebo (n = 12)	Group receiving spironolactone (n = 13)	*P* value
Age (years)	9.4 ±2.0	9.7 ±2.0	0.707
CKCS (yes/ total)	7/ 12	9/ 13	0.688
Sex (male/ total)	7/ 12	11/13	0.202
Body weight (kg)	11.6 ±4.1	11.8 ±6.3	0.940
Systolic blood pressure (mmHg)	156.5 ±29.8	159.4 ±32.0	0.816
Heart rate (ECG) (bpm)	117.3 ±25.0	117.2 ±25.7	0.992
LA:Ao ratio	1.27 ±0.17	1.40 ±0.21	0.105
LVIDdN (cm/[$kg^{0.294}$])	1.79 ±0.20	1.95 ±0.35	0.180
LVIDsN (cm/[$kg^{0.315}$])	1.05 ±0.13	1.10 ±0.28	0.548
LVIDd/ LVFWd ratio	4.06 ±0.63	4.46 ±0.96	0.229
E wave velocity (m/s)	1.01 ±0.22	0.99 ±0.35	0.883
E/ A wave ratio	1.37 ±0.22	1.40 ±0.50	0.857
Serum NT-proBNP (pmol/L)	1006.0 [541.0, 2108.0]	2434.0 [740.0, 3955.0]	**0.005**
Serum cTnI (ng/mL)	0.03 [0.01, 0.16]	0.03 [0.01, 0.19]	0.437
UAC (g/mol)	0.079 [0.044, 0.119]	0.088 [0.042, 0.165]	0.744
PCV (%)	42.7 ±6.4	40.9 ±4.6	0.439
Serum urea (mmol/L)	5.3 ±2.2	6.0 ±1.9	0.426

Serum creatinine (µmol/L)	68.9 [59.7, 146.3]	70.5 [52.5, 130.6]	0.810
Serum Na^+ (mmol/L)	147.7 ±2.5	147.8 ±2.0	0.893
Serum K^+ (mmol/L)	4.2 ±0.4	4.5 ±0.5	0.078
Serum Cl^- (mmol/L)	111.5 ±3.6	113.0 ±2.5	0.237

609

610 All variables in the group receiving placebo included 12 dogs, except UAC (n = 10), E wave
611 velocity (n = 11) and E/A ratio (n = 11). All variables in the group receiving spironolactone
612 included 13 dogs, except UAC (n = 9). The *P* values for variables for which significant
613 between-group differences were detected ($P < 0.05$) are highlighted in bold text.
614 CKCS, cavalier King Charles spaniel; ECG, electrocardiogram; bpm, beats per minute;
615 LA:Ao ratio, left atrial to aortic root ratio; LVIDdN, left ventricular end-diastolic diameter
616 normalized for body weight; LVIDsN, left ventricular end-systolic diameter normalized for
617 body weight; LVIDd/ LVFWd ratio, left ventricular end-diastolic diameter to left ventricular
618 free wall thickness in diastole ratio; NT-proBNP, N-terminal B-type pro-natriuretic peptide;
619 cTnI, cardiac troponin I; UAC, urinary aldosterone to creatinine ratio; PCV, packed cell
620 volume; Na^+, sodium ions; K^+, potassium ions; Cl^-, chloride ions.

621 Table 2: Repeated measures linear mixed model analysis of change over 6 months in
622 variables of primary interest.

Variable	P (between groups)	Unit change in variable per month (B) [95% confidence interval]	SE	P (within group)
Serum NT-proBNP (pmol/L)	0.087			
Placebo group (n = 12)		54.93 [-5.58 to 115.45]	29.22	0.073
Spironolactone group (n = 12)		-18.50 [-78.23 to 41.24]	29.00	0.529
Log (Serum cTnI [ng/mL])	0.708			
Placebo group (n = 12)		-0.010 [-0.035 to 0.015]	0.012	0.420
Spironolactone group (n = 12)		-0.004 [-0.028 to 0.021]	0.012	0.772
LA:Ao ratio	0.110			
Placebo group (n = 12)		0.020 [0.008 to 0.032]	0.006	0.002
Spironolactone group (n = 12)		0.007 [-0.005 to 0.018]	0.006	0.231
LVIDdN ($cm/[kg^{0.294}]$)	0.223			
Placebo group (n = 12)		0.017 [0.006 to 0.029]	0.006	0.005
Spironolactone group (n = 12)		0.007 [-0.004 to 0.019]	0.006	0.194

623

624 Repeated measures linear mixed model analysis of change over 6 months in variables of
625 primary interest (LA:Ao ratio, LVIDdN and serum NT-proBNP and cTnI) for the verum and
626 placebo groups.

627 P (between groups): probability that the rate of change of the variable is different between the
628 group receiving placebo and the group receiving spironolactone.

629 Table 3: Repeated measures linear mixed model analysis of change over 6 months in clinical,
630 echocardiographic and biomarker parameters of secondary interest.

Variable	P (between groups)	Unit change in variable per month (B) [95% confidence interval]	SE	P (within group)
PCV (%)	0.792			
Placebo group (n = 9)		-0.06 [-0.41 to 0.29]	0.17	0.729
Spironolactone group (n = 10)		0.01 [-0.35 to 0.36]	0.17	0.977
Serum urea (mmol/L)	0.406			
Placebo group (n = 12)		0.01 [-0.15 to 0.17]	0.08	0.903
Spironolactone group (n = 12)		0.11 [-0.06 to 0.27]	0.08	0.195
Log (Serum creatinine [µmol/L])	0.223			
Placebo group (n = 12)		-0.001 [-0.006 to 0.004]	0.002	0.706
Spironolactone group (n = 12)		0.003 [-0.002 to 0.008]	0.002	0.177
Serum Na^+ (mmol/L)	0.407			
Placebo group (n = 12)		-0.071 [-0.254 to 0.113]	0.091	0.441
Spironolactone group (n = 12)		0.036 [-0.144 to 0.216]	0.089	0.690
Serum K^+ (mmol/L)	0.328			
Placebo group (n = 12)		0.028 [-0.011 to 0.067]	0.019	0.157
Spironolactone group (n = 12)		0.001 [-0.037 to 0.039]	0.019	0.957
Serum Cl^- (mmol/L)	0.713			
Placebo group (n = 12)		0.023 [-0.258 to 0.306]	0.139	0.871

Spironolactone group (n = 12)		-0.050 [-0.327 to 0.228]	0.138	0.719
Log (UAC[g/mol])	0.223			
Placebo group (n = 9)		0.017 [0.006 to 0.029]	0.006	0.005
Spironolactone group (n = 8)		0.007 [-0.004 to 0.019]	0.006	0.194
Body weight (kg)	0.456			
Placebo group (n = 12)		-0.016 [-0.055 to 0.024]	0.019	0.428
Spironolactone group (n = 12)		-0.036 [-0.074 to 0.002]	0.019	0.066
LVIDsN (cm/[$kg^{0.315}$])	0.839			
Placebo group (n = 12)		0.005 [-0.006 to 0.015]	0.005	0.367
Spironolactone group (n = 12)		0.003 [-0.007 to 0.013]	0.005	0.527
LVIDd/ LVFWd ratio	0.531			
Placebo group (n = 12)		0.053 [-0.005 to 0.029]	0.028	0.070
Spironolactone group (n = 12)		0.028 [-0.029 to 0.086]	0.028	0.315
Log (E wave velocity [m/s])	0.734			
Placebo group (n = 11)		0.001 [-0.006 to 0.009]	0.004	0.740
Spironolactone group (n= 12)		-0.001 [-0.008 to 0.007]	0.003	0.887
E/A ratio	0.876			
Placebo group (n =11)		-0.004 [-0.034 to 0.026]	0.015	0.804
Spironolactone group (n = 12)		-0.007 [-0.035 to 0.022]	0.014	0.625
Heart rate (ECG) (bpm)	0.477			
Placebo group (n = 12)		-0.066 [-1.684 to 1.553]	0.781	0.934
Spironolactone group (n = 12)		-0.858 [-2.449 to 0.733]	0.768	0.276

631

632 Repeated measures linear mixed model analysis of change over 6 months in clinical,

633 echocardiographic and biomarker parameters of secondary interest for the verum and placebo
634 groups.

635 P (between groups): probability that the rate of change of the variable is different between the
636 group receiving placebo and the group receiving spironolactone.

637 P (within group): probability that the rate of change over time within the group = 0.

638 SE, standard error; for definitions of other abbreviations see legend to Table 1.

639 Table 4: Comparisons of selected clinical, echocardiographic and biomarker data between
640 dogs receiving placebo and those receiving spironolactone at the 2 week time point.

641

Variable	Group receiving placebo (n = 10)	Group receiving spironolactone (n = 10)	P value
UAC (g/mol)	0.063 [0.032, 0.077]	0.236 [0.125, 0.339]	**0.006**
PCV (%)	46.0 ±7.8	42.9 ±7.4	0.428
Serum urea (mmol/L)	6.3 ±2.2	6.4 ±2.0	0.925
Serum creatinine (μmol/L)	74.7 [63.4, 93.2]	76.4 [67.7, 83.7]	0.940
Serum Na^+ (mmol/L)	147.1 ±1.7	147.3 ±1.1	0.780
Serum K^+ (mmol/L)	4.5 ±0.2	4.7 ±0.2	0.098
Serum Cl^- (mmol/L)	109.3 ±2.8	111.4 ±3.2	0.144

642 Comparisons of selected clinical, echocardiographic and biomarker data between dogs
643 receiving placebo and those receiving spironolactone at the 2 week time point. All variables
644 in the group receiving placebo included 10 dogs, except PCV and UAC, which each included
645 7 dogs. All variables in the group receiving spironolactone included 10 dogs, except PCV,
646 which included 9 dogs and UAC, which included 7 dogs. The P values for variables for
647 which significant between-group differences were detected ($P < 0.05$) are highlighted in bold
648 text.

649 For definitions of abbreviations see legend to Table 1.

650 Table 5: Comparisons of clinical, echocardiographic and biomarker data between dogs
651 receiving placebo and those receiving spironolactone at the 6 month time point.

Variable	Group receiving placebo (n = 12)	Group receiving spironolactone (n = 12)	P value
Body weight (kg)	11.5 ±4.2	11.5 ±6.9	0.986
Heart rate (ECG) (bpm)	116.8 ±25.8	111.7 ±20.4	0.592
LA:Ao ratio	1.39 ±0.25	1.46 ±0.18	0.427
LVIDdN (cm/[$kg^{0.294}$])	1.89 ±0.24	2.01 ±0.34	0.367
LVIDsN (cm/[$kg^{0.315}$])	1.08 ±0.18	1.11 ±0.28	0.747
LVIDd/ LVFWd ratio	4.37 ±0.82	4.59 ±1.12	0.593
E wave velocity (m/s)	1.02 ±0.19	0.96 ±0.28	0.539
E/ A wave ratio	1.33 ±0.13	1.37 ±0.39	0.722
NT-proBNP (pmol/L)	1188.5 [359.0, 3018.0]	1852.0 [765.0, 4128.0]	**0.051**
cTnI (ng/mL)	0.025 [0.010, 0.150]	0.030 [0.010, 0.170]	0.378
UAC (g/mol)	0.063 [0.010, 0.410]	0.125 [0.060, 0.370]	0.113
PCV (%)	42.8 ±6.3	42.1 ±5.5	0.792
Serum urea (mmol/ L)	5.7 ±1.7	6.7 ±3.5	0.364
Serum creatinine (μmol/L)	76.1 [51.9, 146.3]	82.4 [54.1, 145.5]	0.514
Serum Na^+ (mmol/L)	147.1 ±1.0	147.9 ±1.4	0.127
Serum K^+ (mmol/L)	4.5 ±0.3	4.6 ±0.3	0.448

Serum Cl^- (mmol/L)	111.0 ±2.6	112.5 ±3.6	0.258

Comparisons of clinical, echocardiographic and biomarker data between dogs receiving placebo and those receiving spironolactone at the 6 month time point. All variables in the group receiving placebo included 12 dogs, except UAC (n = 9). All variables in the group receiving spironolactone included 12 dogs, except PCV (n = 11), NT-proBNP (n = 11) and UAC (n =6).

For definitions of abbreviations see legend to Table 1.